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Kinetics of Affinity Labeling the L-Tyrosine/L-Phenylalanine Transport System in *Bacillus subtilis*[1]

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Kinetic analyses of the irreversible inhibition of L-tyrosine and L-phenylalanine transport in *Bacillus subtilis* by phenylalanine chloromethyl ketone revealed that the inhibition was due to an affinity labeling process. Phenylalanine chloromethyl ketone is a competetive inhibitor of L-tyrosine and L-phenylalanine transport. The K_i values for irreversible inhibition of L-tyrosine and L-phenylalanine transport were 194 and 177 μM, respectively, and the first order rate constants for the alkylation reaction leading to inactivation of transport of L-tyrosine and L-phenylalanine were 0.016 and 0.012 min^{-1}, respectively. The similarity of these constants are consistent with the involvement of the same functional site for L-phenylalanine and L-tyrosine transport. A second effect of phenylalanine chloromethyl ketone was inhibition of the uptake of neutral, aliphatic amino acids; transport of basic and acidic amino acids was unaffected by it. Since high concentrations of any amino acid did not reduce the inhibitory effects of phenylalanine chloromethyl ketone on transport of neutral, aliphatic amino acids, an independent effect, not due to an affinity labeling process was inferred. A procedure for selective labeling of the L-tyrosine/L-phenylalanine transport system was demonstrated that should be applicable to the introduction of a radioactive label into the transport protein(s).

An attractive approach to the isolation and characterization of specific transport proteins utilizes the technique of affinity labeling with radioactive reagents (1). The first report of affinity labeling of a transport system involved the use of biotin *p*-nitrophenyl ester to affinity label the highly specific biotin transport system in yeast (2). Later, affinity labeling of the lactose transport system in *Escherichia coli* was reported (3). Most recently, affinity labeling of the glucose transport system in human erythrocyte membranes was investigated (4). The isolation and characterization of the transport proteins involved has not yet been accomplished through the use of radioactive affinity labeling reagents. The problem of random labeling of other membrane proteins through nonspecific reactions, recognized earlier (5) was encountered in the only attempt reported (4). In this communication we report our efforts at developing an affinity labeling method capable of allowing the introduction of a specific label into (a) protein(s) involved in a transport process.

We have interpreted genetic and kinetic evidence (6) as showing that the nonlinear double reciprocal plots of uptake data for

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the specific L-tyrosine/L-phenylalanine transport system of *Bacillus subtilis* are consistent with a homogeneous transport system exhibiting negative cooperativity (7). The evidence we presented in support of this hypothesis is mutually supporting, but indirect. The isolation of the protein(s) involved in a particular transport system would provide an opportunity for a direct and compelling assessment of the transport mechanism.

The L-tyrosine/L-phenylalanine transport system of *B. subtilis* possesses characteristics of specificity and affinity that are suited for the approach of affinity labeling (8). The specificity is for L-amino acids containing a phenyl side chain and a positively charged amino group. Modification of the carboxyl group of either of the natural substrates does not significantly alter binding. Information derived from specificity studies was used to select potential affinity labeling reagents expected to have specificity for the L-tyrosine/L-phenylalanine transport system. Phenylalanine chloromethyl ketone (PCK)[4] (9) and tyrosine chloromethyl ketone (TCK) (8) exhibited the most promising affinity labeling characteristics, but we chose to work with PCK as it is much easier to prepare and is more stable in aqueous solution than TCK. The kinetic analysis of the irreversible inhibition by PCK of L-phenylalanine and L-tyrosine transport, demonstrating that it acts as an affinity labeling reagent for this transport system, is described in this communication. Our data suggest that affinity labeling of the L-tyrosine/L-phenylalanine transport system of *B. subtilis* by radioactive PCK will be possible and may lead to isolation and characterization of (a) transport protein(s) in this system.

MATERIALS AND METHODS

Organism. Bacillus subtilis isolate NP 1, a prototrophic derivative of strain 168 (10), was previously described (11).

Cultivation media. The composition of the minimal salts-glucose (plus trace elements) medium (12) used for growth and a modified formula (lacking nitrogen salts) used for nitrogen starvation were previously described, as was the preparation of frozen cells for use as inocula prior to transport experiments (8).

Preparation of unlabeled and [^3H]3-amino-1-chloro-4-phenyl-2-butanone hydrochloride (PCK and [^3H]PCK). Nominally labeled L-[3-$^3H_2$]phenylalanine (15 Ci/mmol) in 0.1 N HCl, obtained from New England Nuclear (NET-366), was added to 10 mmol L-phenylalanine in 5 ml of 2 N NaOH. [3-^{3}H]Phenylalanine chloromethyl ketone ([^{3}H]PCK) was then synthesized according to the reported procedure for the preparation of phenylalanine chloromethyl ketone (9). PCK and [^{3}H]PCK were further purified by ion-exchange chromatography on SE-Sephadex, a method used previously in the purification of TCK (8), giving a specific activity of 300 μCi/mmol for [^{3}H]PCK. The nmr spectra of labeled and unlabeled PCK were identical.

Uptake protocol. Uptake procedures were as described for L-phenylalanine and L-tyrosine (8) with the exception that the times of incubation with the ^{14}C-labeled amino acids were: 3 min with arginine and aspartic acid; 2 min with methionine; 1 min with leucine, threonine, and alanine; 0.5 min with isoleucine and cystine. The final concentration of each ^{14}C-labeled amino acid was 10 μM unless otherwise indicated in the figure legends.

Uptake of [^3H]PCK. Metabolic inhibitors such as cyanide, azide, DNP, and CCCP could not be used to block transport during affinity labeling of the transport site (up to 2 h of incubation with PCK) since they caused significant cell lysis when used at concentrations giving substantial loss of active transport. Also, DNP and CCCP resulted in irreversible loss of L-tyrosine and L-phenylalanine transport when the cells did not lyse.

[^{3}H]PCK was taken up to the extent of only a few hundred cpm at the highest concentration (1 mM) of the reagent that could be used practicably. (Higher concentrations or incubations longer than 3 min resulted in significant loss of transport activity in the duration of the assay due to affinity labeling.) Thus, we were unable to analyze the kinetics of uptake of PCK.

Specificity of inhibition by PCK. Cultures of cells treated with 1 mM PCK for 2 h under conditions described below for irreversible inhibition showed the same number of viable cells compared to cultures not treated with PCK. Further, the growth rate of whole cells in liquid medium was not measurably altered following PCK treatment (1 mM).

Treatment of B. subtilis with PCK. A 200-ml culture of *B. subtilis* grown as previously described (6) to an absorbance at 600 nm of 0.50, was centrifuged at

[4] Abbreviations used: PCK, phenylalanine chloromethyl ketone; TCK, tyrosine chloromethyketone; DNP, dinitrophenol; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

8000*g* in a Sorvall RC2-B centrifuge for 10 min. The pellet obtained was resuspended in 200 ml of minimal salts-glucose medium lacking nitrogen salts and shaken in a 20°C water bath for 30 min. Chloramphenicol (200 μg/ml, final concentration) was added and the culture was incubated with shaking at 20°C for an additional 30 min. At this time the culture was divided into a number of smaller cultures (volumes are indicated in the appropriate legends) and PCK was added to yield the final concentrations indicated. At each designated time a 5-ml sample of suspended cells was withdrawn, collected on a 45-mm Millipore membrane filter (0.45 μm pore size), washed with 100 ml of nitrogen-deficient minimal medium, and finally resuspended in 5 ml of this same medium containing 0.5% glucose and 200 μg/ml chloramphenicol. The uptake of ^{14}C-labeled L-amino acid was then assayed.

Chemicals. Uniformly ^{14}C-labeled L-amino acids having the following specific activities (mCi/mmol) were obtained from New England Nuclear: tyrosine (469), leucine (311), arginine (311), aspartate (207), isoleucine (250), alanine (157), threonine (208), cystine (255), and L-[*methyl*-^{14}C] methionine (41.3). Uniformly labeled L-[^{14}C] phenylalanine in 50% ethanol (492 mCi/mmol) was purchased from Amersham/Searle. L-Phenylalanine, L-leucine, L-threonine, L-isoleucine, L-methionine, L-alanine, CCCP, and chloramphenicol were products of Sigma Chemical Corp. L-Tyrosine was obtained from Calbiochem. All other chemicals were reagent grade.

RESULTS AND DISCUSSION

Competitive inhibition of L-*tyrosine and* L-*phenylalanine transport by PCK.* PCK was shown to be a good inhibitor of L-phenylalanine or L-tyrosine uptake at 37°C (8). An affinity labeling reagent binds reversibly at the site for which it is specific prior to its covalent attachment to the site. Figure 1A and B show that PCK is a competitive inhibitor of L-tyrosine and L-phenylalanine uptake at 20°C over the entire concentration ranges that span the negative cooperative uptake kinetics defined by substrate saturation curves (6). The break in the curve simulates a biphasic plot. For convenience the concentration ranges on either side of the break are denoted low-affinity phase and high-affinity phase although the true kinetic constants presumably vary continuously within each phase as expected of the negative cooperative system (7).

Dissociation constants, K_i, for competitive inhibition by PCK were determined from the slopes and intercepts obtained from each of the two arbitrarily defined phases. The high-affinity phase of uptake exhibited K_i values of 121 and 190 μM for L-phenylalanine and L-tyrosine, respectively. The low-affinity phase of uptake exhibited K_i values of 80 and 115 μM for L-phenylalanine and L-tyrosine, respectively. These K_i values are very similar; the differences could be due to variations in binding constants of multiple sites in a putative negative cooperative system in-

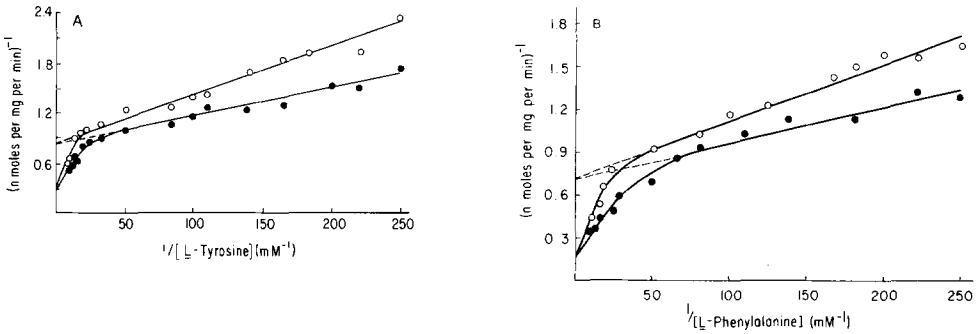

FIG. 1. Kinetics of L-tyrosine uptake (A) or L-phenylalanine uptake (B) into whole cells of *B. subtilis* in the presence or absence of PCK. Uptake velocity is expressed on the ordinate scale as nmoles of L-[^{14}C]tyrosine or L-[^{14}C] phenylalanine taken up per milligram of cell (dry weight) per minute at 20°C and is plotted as a function of the reciprocal of L-[^{14}C]tyrosine concentration (A) or L-[^{14}C]phenylalanine concentration (B) in the absence (●) or presence (○) of 100 μM PCK. Apparent V values for L-tyrosine entry (A) were 1.2 and 3.3 nmol/mg (dry weight)/min and the K_i values were 190 and 115 μM for the high- and low-affinity systems, respectively. The apparently V values for L-phenylalanine entry (B) were 1.4 and 6.8 nmol/mg (dry weight)/min and the K_i values were 121 and 80 μM for the high- and low-affinity systems, respectively.

teracting with PCK and either L-tyrosine or L-phenylalanine.

Uptake of PCK. Although PCK is an effective inhibitor of aromatic amino acid transport, it is itself transported poorly by *B. subtilis*. Since PCK is an irreversible inhibitor of the transport system to which PCK binds, longer assay periods or higher concentrations of PCK during the assay could not be used. Thus, we were unable to analyze the kinetics of uptake of PCK.

In order to restrict the alkylating effect of PCK to the exterior of the cells during irreversible inhibition experiments taking up to 2 h, we investigated the abilities of metabolic inhibitors to block transport. At concentrations of cyanide, azide, DNP, or CCCP giving substantial loss of active transport, significant cell lysis was usually observed. The effects of DNP and CCCP upon transport were largely irreversible when cell lysis was not observed.

Since cells treated with PCK for 2 h showed the same number of colonies as a control incubated for the same time in the absence of PCK, it appears that alkylation of intracellular constituents by PCK causing lethality is not significant. The growth rate of cells pretreated with PCK was similar to an untreated control. Thus, the inhibitory effects of PCK were not secondarily due to a general effect on vital processes essential to viability and active transport.

Irreversible inhibition of L-tyrosine and L-phenylalanine uptake by PCK. Incubation of nitrogen-starved, chloramphenicol-treated cells at 20°C with 10–1000 μM PCK resulted in a progressive, irreversible inhibition of L-tyrosine (Fig. 2A) or L-phenylalanine (Fig. 2B) uptake. Inhibition of transport by PCK could not be reversed by extensive washing or prolonged incubation of washed PCK-treated cells in fresh transport medium.

Since PCK inactivated L-tyrosine or L-phenylalanine transport competitively and irreversibly, the mechanism of inhibition can be represented as follows:

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \xrightarrow{k_3} EI'; K_i = \frac{(E)\,(I)}{(EI)}$$

where E is the transport component that binds PCK, I is PCK, EI is a reversible

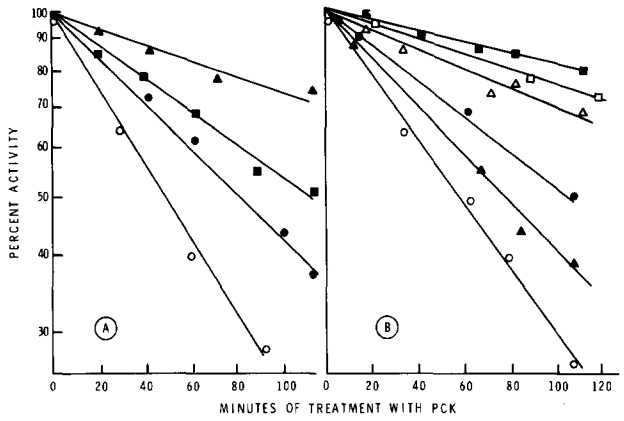

FIG. 2. Inactivation of L-tyrosine (A) or L-phenylalanine (B) uptake as a function of time at various concentrations of PCK. Nitrogen-starved, chloramphenicol-treated cell samples (30 ml) were incubated with various concentrations of PCK for the designated lengths of time at 20°C. At the indicated times, 5-ml samples were withdrawn from the shaking culture, washed with 100 ml of nitrogen-deficient medium, and resuspended in 5 ml of the nitrogen-deficient medium containing 200 μg/ml chloramphenicol and 0.5% glucose. Assay of L-[^{14}C]tyrosine and L-[^{14}C]phenylalanine transport and other procedural details are described in Methods. The rate of transport of ^{14}C-labeled L-amino acid in untreated cell cultures is denoted as 100% activity. Symbols: (A) inactivation of L-tyrosine uptake by PCK concentrations of 1000 μM(○); 440 μM(●); 100 μM(■); and 55 μM(▲): (B) inactivation of L-phenylalanine uptake by PCK concentrations of 1000 μM(○); 500 μM(▲); 200 μM(■); 100 μM(△); 50 μM(□); and 40 μM(■).

complex, and EI′ is an irreversible (covalent) complex. This is the kinetic analysis that has been used to described the irreversible inhibition of acetylcholinesterase by esters of methanesulfonic acid (13). This treatment results in the equation: $\ln [(E_T\text{-}EI')/E_T] = -k_{app} \cdot t$, where $E_T = E + EI + EI'$, $k_{app} = k_3 I/(I + k_i)$, and $\ln[(E_T - EI')/E_t]$ is the percent of transport activity remaining after treatment of cell cultures with PCK as described in Materials and Methods.

The rate of inactivation of L-tyrosine and L-phenylalanine transport by PCK was observed to be psuedo first-order (Fig. 2). Double reciprocal plots (Fig. 3) of the apparent rate constants of inactivation (k_{app}) (determined from the slopes of the inactivation curves shown in Fig. 2) against the concentrations of PCK resulted in straight lines with intercepts on the ordinate. These data suggest that the inactivation of L-tyrosine and L-phenylalanine transport by PCK occurs via a reversible (EI) complex in accordance with the above mechanism for irreversible inhibition. The first-order rate constants for inactivation of transport by PCK, k_3, determined from the intercepts ($1/k_3$) of these plots were 0.016 and 0.012 min^{-1} for L-tyrosine and L-phenylalanine uptake (Fig. 3), respectively. The dissociation constants, K_i, determined from the slopes (K_i/k_3) of the lines plotted in Fig. 3, were 194 and 177 μM for inhibition by PCK of L-tyrosine and L-phenylalanine uptake, respectively. The good agreement between these constants indicates that the same site is involved in the binding of PCK (K_i) and that the same chemical reaction is occurring in the inactivation process (k_3). This supports our proposal (6, 8) that the nonlinear kinetics of L-tyrosine and L-phenylalanine transport is due to a homogeneous transport system exhibiting negative cooperativity.

Substrate protection in the inactivation of L-tyrosine and L-phenylalanine uptake by PCK. If irreversible inhibition by PCK occurs by an affinity labeling process, either amino acid substrate should reduce the rate of inactivation by PCK. As shown in Table I, the presence of either 1 mM L-tyrosine or 1 mM L-phenylalanine during treatment of cells with 1 mM PCK significantly reduces the amount of inactivation by PCK of the L-tyrosine/L-phenylalanine transport system.

Specificity of inhibition by PCK. The effect of PCK on the uptake of other amino acids possessing acidic, basic, or neutral

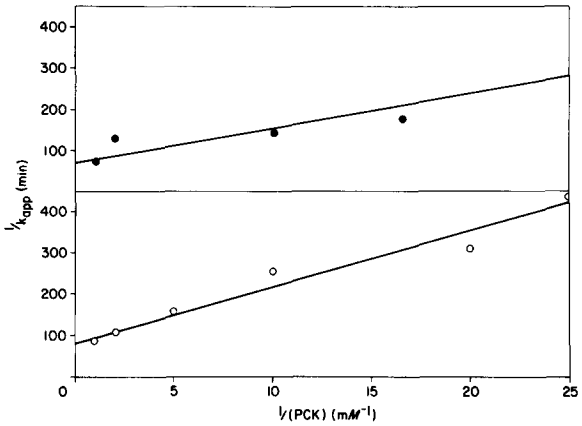

FIG. 3. Saturation kinetics of PCK inactivation of L-tyrosine (top) or L-phenylalanine (bottom) uptake. The reciprocal of the apparent rate of inactivation, k_{app}, obtained from the slopes of the lines shown in Fig. 2 (expressed in minutes), is plotted as a function of the reciprocal of PCK concentration. The ordinate intercept and the slope provide the values of $1/k_3$ and K_i/k_3, respectively, where k_3 is the first-order rate constant for inactivation of the transport component (12). The K_i and k_3 values for PCK inactivation of L-tyrosine transport were 194 and 0.016 reciprocal minutes, respectively. The K_i and k_3 values for inactivation of L-phenylalanine transport by PCK were 177 μM and 0.012 reciprocal minutes, respectively.

TABLE I

EFFECT OF PCK[a] ON AMINO ACID TRANSPORT

Transport substrate[b]	Amino acid present 1 mM	Percent inhibition[c]
L-Phenylalanine	None	80
	L-Phenylalanine	39
	L-Tyrosine	38
	L-Leucine	73
L-Tyrosine	None	75
	L-Tyrosine	38
	L-Phenylalanine	39
	L-Leucine	60
L-Leucine	None	90
	L-Leucine	89
	L-Phenylalanine	85
	L-Tyrosine	86
L-Threonine	None	88
	L-Threonine	86
L-Isoleucine	None	74
	L-Isoleucine	75
L-Methioine	None	87
	L-Methionine	89
L-Alanine	None	60
	L-Alanine	67
L-Arginine	None	12
L-Aspartate	None	19
L-Cystine	None	6

[a] The final concentration of PCK was 1 mM.

[b] The concentration and time of uptake of each ^{14}C-labeled L-amino acid used is described in Materials and Methods.

[c] Cell cultures were treated with PCK for 90 min, filtered, washed and resuspended in transport medium as described in Fig. 2. Percent inhibition is expressed as percentage of transport activity compared to untreated control cultures.

side chains was determined. As demonstrated in Table I, L-arginine, L-aspartate, and L-cystine transport were only slightly affected by PCK. However, the transport of L-leucine, L-isoleucine, L-alanine, L-methionine, and L-threonine (Table I) was irreversibly inhibited by PCK. High concentrations of L-leucine, L-tyrosine, and L-phenylalanine, did not reduce the rate of inactivation of L-leucine transport caused by PCK (Table I). Likewise, the presence of L-isoleucine, L-alanine, L-methionine, or L-threonine did not protect against inactivation of neutral, aliphatic amino acid transport by PCK (Table I). High concentrations of L-leucine, a non competitive inhibitor of the L-tyrosine/L-phenylalanine transport system (8) did not reduce the rate of inactivation of L-phenylalanine or L-tyrosine transport caused by PCK. On the other hand, L-tyrosine and L-phenylalanine significantly reduced the inhibition of L-tyrosine and L-phenylalanine transport caused by PCK (Table I). Therefore, PCK, in addition to acting as an active site-directed irreversible inhibitor specific for the L-tyrosine/L-phenylalanine transport system, alkylates some component(s) that influences the transport of neutral, aliphatic amino acids. This second effect does not fulfill criteria expected of an affinity labeling process.

Conditions for selective labeling of the L-tyrosine/L-phenylalanine transport system by PCK. While PCK irreversibly inhibits the transport of neutral, aliphatic amino acids in addition to affinity labeling of the specific L-tyrosine/L-phenylalanine transport system, it should be possible to selectively label the L-tyrosine/L-phenylalanine transport system with [^{3}H]PCK if the cells are first pretreated with PCK in the presence of high concentrations of L-phenylalanine or L-tyrosine. This would allow selective inactivation of the transport system(s) for neutral, aliphatic amino acids by PCK treatment while the specific transport system is protected by the substrate. Subsequent treatment of the cells with [^{3}H]PCK in the absence of substrate would affinity label the previously protected L-tyrosine/L-phenylalanine transport system with [^{3}H]PCK.

The feasibility of this is demonstrated in Fig. 4. Cells were incubated with 1 mM PCK in the presence of 50 mM L-phenylalanine resulting in a rapid loss (80%) of L-leucine transport capability while only 20% of L-phenylalanine transport was lost. The cells were filtered, washed, and resuspended in fresh medium containing PCK. The remaining transport activities for L-leucine and L-phenylalanine were now rapidly lost, indicating the preferential protection by L-phenylalanine of the transport system for which it was a substrate.

Application of PCK as an affinity labeling reagent. We have presented a kinetic

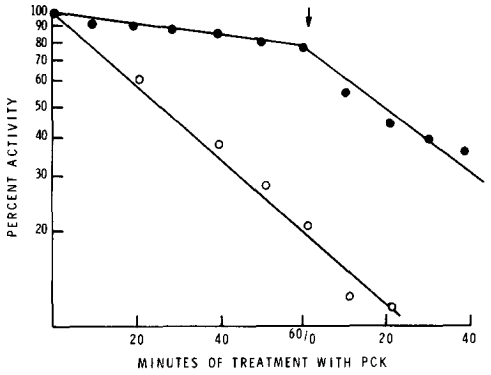

FIG. 4. Inactivation of L-phenylalanine and L-leucine uptake by PCK in cells protected by L-phenylalanine. A 100 ml-culture of nitrogen-starved, chloramphenicol-treated cells was incubated with 50 mM L-phenylalanine for 30 min at 20°C. PCK was then added to a final concentration of 1 mM. At the indicated times, 5-ml samples were withdrawn from the shaking culture, washed and resuspended in the nitrogen-deficient medium containing chloramphenicol. L-Phenylalanine (●) and L-leucine (○) transport rates were assayed. After 60 min of this treatment, the cells were collected by centrifugation at 7000*g*, washed with 100 ml of the nitrogen-deficient medium containing chloramphenicol and resuspended in 100 ml of this medium. PCK (500 μM final concentration) was added this time in the absence of L-phenylalanine, and the amount of irreversible inhibition of L-phenylalanine and L-leucine transport was determined as described above. Other procedural details are described in Fig. 2 and Methods.

analysis consistent with the role of PCK as an affinity labeling reagent for the L-tyrosine/L-phenylalanine transport system: 1. PCK binds competetively with L-tyrosine and L-phenylalanine. 2. The inactivation of L-tyrosine and L-phenylalanine transport by PCK is saturable and proceeds via a kinetically demonstrable transport site–PCK complex. 3. The natural substrates for the transport system protect against the effects of PCK while other nonsubstrate amino acids such as L-leucine have no effect. 4. While PCK does inactivate the transport of neutral, aliphatic amino acids, it cannot be an affinity labeling process since the natural substrates do not protect against PCK.

In order to selectively affinity label the L-tyrosine/L-phenylalanine transport system with [^{3}H]PCK it will be necessary to inactivate the uptake of neutral, aliphatic amino acids with unlabled PCK while the L-phenylalanine transport system is protected by substrate. Thus, our kinetic studies show that we can selectively label the L-tyrosine/L-phenylalanine transport system and suggest that we will be able to use [^{3}H]PCK to selectively label the L-tyrosine/L-phenylalanine transport system. This label will aid in the isolation of a transport component(s) allowing us to characterize it chemically and physically.

REFERENCES

1. BAKER, B. R. (1967) Design of Active-Site-Directed Irreversible Enzyme Inhibitors, Wiley, New York.
2. BECKER, J. M., WILCHEK, M., AND KATCHALSKI, E. (1971) *Proc. Nat. Acad. Sci. USA* **68,** 2604–2607.
3. YARIV, J., KALB, A. J., AND YARIV, M. (1972) *Fed. Eur. Biochem. Soc. Lett.* **27,** 27–29.
4. TAVERNA, R. D., AND LANGDON, R. G. (1972) *Biochem. Biophys. Res. Comm.* **54,** 593–599.
5. CHAVIN, S. I. (1971) *Fed. Eur. Biochem. Soc. Lett.* **14,** 269–282.
6. GLOVER, G. I., D'AMBROSIO, S. M., AND JENSEN, R. A. (1975) *Proc. Nat. Acad. Sci. USA*, in press.
7. LEVITZKI, A., AND KOSHLAND, D. E. (1969) *Proc. Nat. Acad. Sci. USA* **62,** 1121–1128.
8. D'AMBROSIO, S. M., GLOVER, G. I., NELSON, S. O., AND JENSEN, R. A. (1973) *J. Bacteriol.* **115,** 673–681.
9. SHAW, E., AND RUSCICA, J. (1971) *Arch. Biochem. Biophys.* **145,** 484–489.
10. ARMSTRONG, R. L., HARFORD, N., KENNETT, R. H., ST. PIERRE, M. L., AND SUEOKA, N. (1970) *Methods Enzymol.* **17 A,** 36–59.
11. KANE, J. F., STENMARK, S. L., CALHOUN, D. H., AND JENSEN, R. A. (1971) *J. Biol. Chem.* **246,** 4308–4316.
12. JENSEN, R. A. (1968) *Genetics* **60,** 707–717.
13. KITZ, R., AND WILSON, I. B. (1962) *J. Biol Chem.* **237,** 3245–3249.